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Biochemical Analysis of Metallo-β-Lactamase NDM-3 from a Multidrug-Resistant *Escherichia coli* Strain Isolated in Japan

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New Delhi metallo-β-lactamase-3 (NDM-3) was identified in a multidrug-resistant *Escherichia coli* isolate, NCGM77, obtained from the feces of a patient in Japan. The enzymatic activities of NDM-3 against β-lactams were similar to those of NDM-1, although NDM-3 showed slightly lower k_{cat}/K_m ratios for all the β-lactams tested except for doripenem. The genetic context for bla_{NDM-3} was *tnpA*-bla_{NDM-3}-ble_{MBL}-*trpF-dsbC-tnpA-sulI-qacEdeltaI-aadA2-dfrA1*, which was present on an approximately 250-kb plasmid.

Metallo-β-lactamases (MBLs) are produced by many species of Gram-negative bacteria and some species of Gram-positive bacteria, including *Bacillus* spp. (1, 2). MBLs can confer resistance or reduced susceptibility to carbapenems and, usually, to cephalosporins and to penicillins except for monobactams (3). New Delhi metallo-β-lactamase-1 (NDM-1), a recently discovered MBL, was initially found in Sweden from *Klebsiella pneumoniae* and *Escherichia coli* isolates that originated from India (4). Subsequently, at least 10 NDM variants (see www.lahey.org/studies) have been reported in several different countries (5–17).

Escherichia coli NCGM77 was isolated from the feces of a patient in a medical setting in Japan in 2013. The isolate was phenotypically identified, and species identification was confirmed by 16S rRNA sequencing (18). MICs were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (19). *E. coli* NCGM77 was resistant to various tested β-lactams (Table 1); the MICs of the other antibiotics were 32 μg/ml (amikacin), 8 μg/ml (arbekacin), 256 μg/ml (ciprofloxacin), <0.25 μg/ml (colistin), >1,024 μg/ml (fosfomycin), 64 μg/ml (gentamicin), 512 μg/ml (kanamycin), 32 μg/ml (levofloxacin), 1 μg/ml (minocycline), <0.25 μg/ml (tigecycline), and 64 μg/ml (tobramycin). PCR analysis for MBL genes of bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM} was performed (20, 21). On the basis of the PCR results, the isolate was positive for bla_{NDM}. The DNA sequence of the PCR product revealed that the isolate had the bla_{NDM-3} gene (9). Multilocus sequence typing (MLST) of NCGM77 found it to be sequence type 88 (ST88) (*E. coli* MLST database [see http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html]). *Pseudomonas aeruginosa* IOMTU9 (17) was used as a source of the bla_{NDM-1} gene.

The bla_{NDM-3} and bla_{NDM-1} genes were cloned into the corresponding sites of pHSG398 (TaKaRa, Shiga, Japan) using the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′). The *Escherichia coli* DH5α strain was transformed with pHSG398-NDM-3 or pHSG398-NDM-1 to determine the MICs of β-lactams.

The open reading frames of NDM-1 and NDM-3 without signal peptide regions were cloned into the pET28a expression vector (Novagen, Inc., Madison, WI) using the primer set BamHI-TEV-NDM-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCCAGCAAATGGAAACTGGCGAC-3′) and XhoI-NDM-R (5′-ATCTCGAGTCAGCGCAGCTTGTCGGCCATG-3′). Plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA). Recombinant NDM proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instructions (Qiagen, Hilden, Germany). His tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA), and untagged proteins were purified by an additional passage over the Ni-NTA agarose. The purities of NDM-1 and NDM-3 were >90%, as estimated by SDS-PAGE. During the purification procedure, the presence of β-lactamase activity was monitored using nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM phosphate buffer (pH 7.0) containing 5 μM $Zn(NO_3)_2$ at 37°C, using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratios were determined by analyzing β-lactam hydrolysis using a Lineweaver-Burk plot. Wavelengths and extinction coefficients for β-lactam substrates have been reported previously (22–24). Three individual experiments were performed to determine the K_m and k_{cat} values.

All cloned genes in the pHSG398 and pQE2 vectors were sequenced using an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA).

The plasmid harboring bla_{NDM-3} was extracted (25) and sequenced by MiSeq (Illumina, San Diego, CA). The size of the plasmid harboring bla_{NDM-3} was determined using pulsed-field gel electrophoresis (PFGE) and Southern hybridization (12). A probe for bla_{NDM-3} was amplified by PCR by using the EcoRI-NDM-F and the PstI-NDM-R primers. Signal detection was carried out using the DIG High Prime DNA labeling and detection starter kit II (Roche Applied Science, Indianapolis, IN).

The bla_{NDM-3} probe hybridized to a 250-kb plasmid (Fig. 1). The sequence surrounding bla_{NDM-3} was *tnpA*-bla_{NDM-3}-ble_{MBL}-*trpF-dsbC-tnpA-sulI-qacEdeltaI-aadA2-dfrA1*. This plasmid showed more than 99.9% identity at the nucleotide sequence level from

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TABLE 1 MICs of various β-lactams for *E. coli* strains NCGM77 and DH5α transformed with plasmids carrying NDM-1 or NDM-3

Antibiotic	MIC (μg/ml) for:			
	NCGM77	pHSG398/ NDM-3	pHSG398/ NDM-1	pHSG398
Ampicillin	>1,024	256	256	4
Ampicillin-sulbactam	>1,024	128	128	2
Aztreonam	>1,024	0.063	0.063	0.063
Cefepime	>1,024	0.25	0.5	0.063
Cefoselis	—[a]	2	4	0.031
Cefotaxime	>1,024	4	8	0.031
Cefoxitin	>1,024	32	64	4
Cefpirome	—[a]	1	0.5	0.015
Ceftazidime	>1,024	256	256	0.25
Ceftriaxone	—[a]	8	8	0.031
Cephradine	>1,024	256	256	16
Doripenem	32	0.125	0.125	0.031
Imipenem	16	0.25	0.5	0.063
Meropenem	32	0.25	0.5	<0.015
Moxalactam	—[a]	8	8	0.125
Penicillin G	>1,024	128	256	32

[a] —, MICs of cefoselis, cefpirome, ceftriaxone, and moxalactam for the NCGM77 strain were not determined.

TABLE 2 Kinetic parameters of NDM-3 and NDM-1 enzymes[a]

β-Lactam	NDM-3			NDM-1		
	K_m[b] (μM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Ampicillin	228 ± 35	73 ± 7	0.32	500 ± 14	255 ± 8	0.51
Aztreonam	NH[c]	NH[c]	NH[c]	NH[c]	NH[c]	NH[c]
Cefepime	103 ± 10	17 ± 1	0.16	147 ± 18	41 ± 3	0.28
Cefotaxime	28 ± 3	24 ± 1	0.9	36 ± 4	63 ± 2	1.7
Cefoxitin	17 ± 1	1.6 ± 0.2	0.10	20 ± 3	4.4 ± 0.2	0.22
Ceftazidime	64 ± 9	11 ± 1	0.17	233 ± 35	58 ± 5	0.25
Cephradine	12 ± 3	26 ± 1	2.2	13 ± 2	66 ± 1	5.0
Doripenem	92 ± 2	34 ± 1	0.37	116 ± 18	41 ± 4	0.35
Imipenem	148 ± 13	25 ± 1	0.17	123 ± 21	59 ± 3	0.48
Meropenem	81 ± 4	32 ± 1	0.40	78 ± 6	74 ± 2	0.95
Penicillin G	42 ± 3	47 ± 1	1.1	24 ± 4	99 ± 4	4.3

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] K_m and k_{cat} values represent the means of 3 independent experiments ± the standard deviations.
[c] NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

69,229 to 78,275 bp of the pGUE plasmid (GenBank accession no. JQ364967) from the *E. coli* strain GUE, which was isolated in India (26). The entire sequence of the plasmid was not determined with the sequence data generated by MiSeq (Illumina).

Expression of the bla_{NDM-3} and bla_{NDM-1} genes in *E. coli* DH5α conferred resistance or reduced susceptibility to all cephalosporins, moxalactam, and carbapenems (Table 1). The MIC of cefpirome was 2-fold higher for *E. coli* expressing NDM-3 than for *E. coli* expressing NDM-1. In contrast, those of cefepime, cefoselis, cefotaxime, cefoxitin, imipenem, meropenem, and penicillin G were 2-fold lower for NDM-3 than for NDM-1.

As shown in Table 2, recombinant NDM-3 and NDM-1 hydrolyzed all β-lactams tested except for aztreonam. The profiles of the enzymatic activities of NDM-3 against β-lactams tested were similar to those of NDM-1, although NDM-3 had slightly but significantly lower k_{cat}/K_m ratios for all β-lactams tested except for doripenem. The lower k_{cat}/K_m ratios were mostly caused by the lower k_{cat} values of NDM-3 compared with those of NDM-1, i.e., the values of NDM-3 were 19.0 to 47.5% of those of NDM-1 (Table 2). In fact, the substitution from Asp to Asn at position 95 of NDM appeared to decrease the hydrolysis rate of all β-lactams tested except for doripenem (Table 2). An amino acid substitution at position 95 from Asp to Asn decreased the k_{cat} values of NDM-3 compared to those of NDM-1 (Table 2). Residue 95 is in α1, which is located on the protein surface. The crystal structure of NDM-1 revealed that the active site of NDM-1 is located at the bottom of a shallow groove enclosed by 2 important loops, L3 and L10 (27–30). Residue 95 in α1, however, was not located in these loops. This residue may indirectly affect the interaction of the substrate with the active site. Among all 9 NDM variants, amino acid substitutions were identified at 7 positions (28, 88, 95, 130, 152, 154, and 233). It remains unclear which position(s) plays a critical role in the enzymatic activities. Relative to IMP-1 and VIM-2, NDM-1 does not bind to carbapenems as tightly, but it turns over carbapenems at a rate similar to that of VIM-2 (4). NDM-4 with an amino acid substitution at position 154 (Met to Leu) showed greater hydrolytic activity toward carbapenems, cephalotin, cefotaxime, and ceftazidime than that shown by NDM-1 (12). NDM-5 with substitutions at positions 88 (Val to Leu) and 154 (Met to Leu) resulted in reduced susceptibilities of *E. coli* transformants to cephalosporins and carbapenems (12). NDM-8 with substitutions at positions 130 (Asp to Gly) and 154 (Met to Leu) resulted in enzymatic activities against β-lactams that were similar to those of NDM-1 (17). The drug susceptibilities of *E. coli* transformants with bla_{NDM-2}, bla_{NDM-3}, bla_{NDM-6}, bla_{NDM-7}, and bla_{NDM-9} have not been reported.

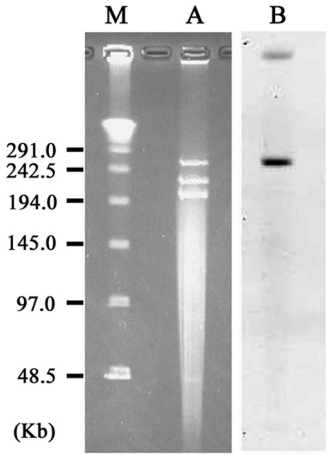

FIG 1 Localization of the bla_{NDM-3} gene on the plasmid of *E. coli* strain NCGM77 separated by PFGE. Lane M, midrange PFG marker (New England BioLabs, Tokyo, Japan); lane A, plasmids of *E. coli* strain NCGM77; lane B, hybridization of the plasmid with a probe specific for the bla_{NDM-3} gene.

This is the first report describing NDM-3-producing Gram-negative pathogens in Japan. It appears that NDMs have recently begun evolving; therefore, careful monitoring of NDM-producing pathogens is required.

Plasmid sequence accession number. The plasmid sequence including the bla_{NDM-3} gene has been deposited in GenBank under the accession number AB898038.

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